STUDIES OF FERTILIZATION.

VII. Analysis of Variations in the Fertilizing Power of Sperm Suspensions of Arbacia.

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I. Introduction.

In his epoch-making "Expériences pour servir a l'histoire de la génération des animaux et des plantes" published in 1785 the Abbé Spallanzani describes among his numerous experiments on fertilization and artificial parthenogenesis some determinations concerning the minimal quantity of sperm necessary to fertilize the eggs of the frog. He found that he could get perfect fertilization with seminal fluid diluted 2,720 times with water. At greater dilutions the percentage of fertilized eggs began to fall off, but some eggs fertilized up to a dilution of about 20,000 times. He calculated that the weight of the "spermatic particles" necessary to fertilize an egg was 1/2,994,687,500 of a grain, and that the volume of the egg in proportion to the volume of spermatic particles necessary to fertilize it is as 1,064,777,777: 1. In 1824 Prevost et Dumas confirmed these calculations.

So far as I know such experiments have not been repeated. The reason for this would appear to be that when it was once established that only a single spermatozoön unites with each ovum in fertilization all such quantitative studies of the fertilizing power of sperm dilutions appeared to have lost their point. So long as it was assumed (as was generally the case) that the fertilizing power of the spermatozoön is a function of its motility

alone, that is, of its capacity to "penetrate the ovum," there could be no object in quantitative studies. But as it came to be recognized that the fertilizing power of the spermatozoön is associated with some definite substance that it bears, possibly either a lysin (Loeb) or an activator (self), the problem assumes a different aspect; for it is obvious that if the sperm should lose such a substance in any way, its fertilizing power would be lost even though its motility should be preserved unimpaired. In such a case the relative fertilizing power of sperm suspensions could not be measured either in terms of concentration or of activity of the spermatozoa. Variations in the fertilizing power of suspensions of known concentrations might, therefore, be a measure of the loss of the postulated fertilizing substance. On reflection it is obvious that the spermatic substance in question must be loosely bound to the sperm, because it exerts its first effect, that of inducing cortical changes in the egg, before penetration, as I have shown for *Nereis*, and Loeb for certain hybrid combinations; at this time, therefore, the spermatozoön must set free its receptors[1] (activators).

Recently Glaser (1913 and 1915) has maintained that in *Arbacia* more than one spermatozoön is needed for fertilization of the egg, even though only one actually penetrates. The observations on which this conclusion rests are no doubt correct, *under the given conditions*, and I have made similar observations, as will appear in the course of the present paper. But it by no means follows from the observations that a single spermatozoön may not be adequate under other conditions (and this can be demonstrated). We cannot, however, deny *a priori* the possibility that for the initial phases of fertilization a number of spermatozoa may be of assistance though only one enters and is concerned in later phases. If the phenomena of fertilization are to receive a physiological, and ultimately a chemical, inter-

[1] In study VI. (1914), I propounded a theory of fertilization according to which the initiation of development of the egg is due to activation of an ovogenous substance, which I named fertilizin, contained in the cortex of the egg. In fertilization such activation is caused by a certain constituent of the sperm, which I called the sperm receptors; and the action of the fertilizin thus aroused must be on certain substances of the egg which I named in general egg-receptors. From a chemical point of view therefore we must have an interaction of three substances (or groups of substances), viz., sperm receptors, fertilizin, and egg receptors.

pretation, quantitative questions may be of serious significance.

It would seem to be a perfectly simple matter to determine the greatest dilution of sperm at which any fertilization takes place, and to express in the form of a curve, from percentages of eggs fertilized, the rate of loss of fertilizing power due to dilution. This was the very simple problem with which the present investigation began. However the results were in the highest degree contradictory; the same lot of sperm might vary in a period of half an hour from 1/1,024 to 1/9,000,000 (or less) of 1 per cent. dilution in its power to fertilize the same percentage of a single lot of eggs. The investigation, therefore, turned to the problem of such variations and their cause.

II. Experiments.

1. *Methods.*

Quantitative methods cannot possibly be as rigorous in a problem of this kind as in a purely chemical problem. In the first place we have to deal with variable reagents in the ova and sperm of *Arbacia;* and in the second place the initial measurements must be made rather hurriedly, so as to ensure freshness of the reagents, and under conditions that do not injure their vitality; the available quantities of material also limit the methods of measurement.

Sperm.—The standard for measurements of sperm dilutions is the "dry sperm"; *i. e.*, the thick creamy mass that exudes from ripe testes of *Arbacia.* If a ripe male be opened and inverted in a dry Syracuse watch crystal a certain amount entirely free from foreign admixture usually flows from the genital pores and collects in a mass in the crystal. While this may in certain cases be as much as 2 c.c., usually it is a much smaller quantity. It is quite impracticable to measure this by graduated pipettes; I have therefore used a drop of this dry sperm from bulb pipettes of fairly uniform openings as a standard, and, reckoning 30 such drops to the cubic centimeter, have made "1 per cent. sperm suspensions" by the addition of such a drop to 3.3 c.c. of sea water. This is the standard suspension from which most of the experiments proceed, and all sperm suspensions are expressed in fractions of such a 1 per cent. suspension. Given perfectly

dry sperm, the initial variation due to the method cannot be very great in relation to the tremendous range of variation in fertilizing capacity of sperm due to other causes. Indeed it is a vanishing quantity.

Eggs.—Egg concentration is a factor of relatively slight significance within the limits of the experiments, as will appear from the facts to be presented. Within a very wide range it does not affect the result measured in percentage of fertilized eggs. It is measured roughly by allowing washed eggs to settle for half an hour in a 100-c.c. graduated cylinder, and expressing the quantity there settled as a percentage of the entire fluid. There is of course for every concentration of sperm an egg concentration that is above the optimum for percentage of fertilization. But, as will be seen from the tables, such egg concentration lies beyond the concentration used in most experiments.

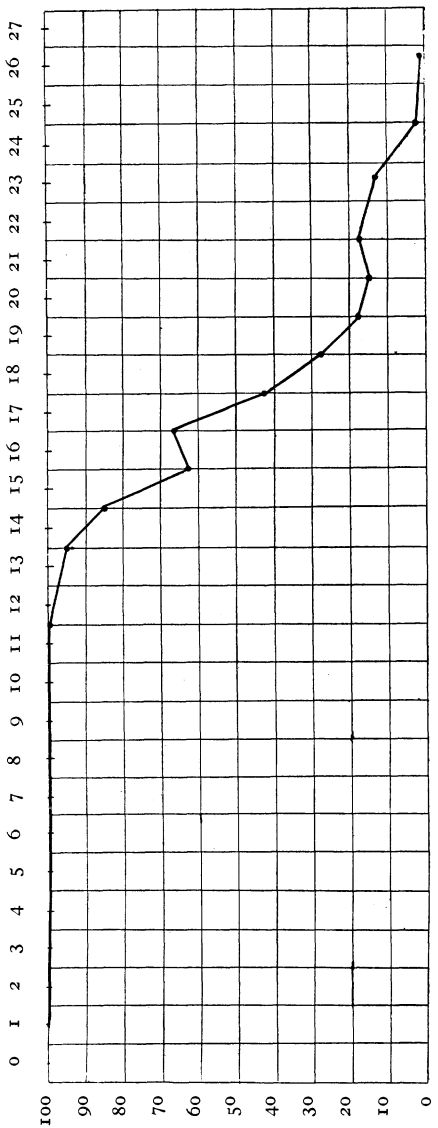
In most cases segmentation of the eggs was used as the criterion of fertilization, but membrane formation was also used in some cases, especially in high concentrations of sperm where many eggs failed to segment owing to polyspermy.

Formulation of Results.—The fertilizing power of sperm suspensions is expressed in curves whose ordinates are percentages of fertilization, and the abscissæ a geometrical series of dilutions of 1 per cent. sperm in powers of 2. This method was adopted for the abscissæ because of the method of successive half dilutions used in many experiments, and because the enormous range of fertilizing power made it impossible to compare results on one scale with an arithmetical progression. When it is realized that the fertilizing power may cease at 1/156 of 1 per cent., or extend to 1/90,000,000 the necessity of the geometrical series in the abscissæ will become apparent.

2. *The Optimum Curve of Dilutions.*

We may begin with the optimum curve of dilutions (Curve 1), because this answers most completely, and probably fully, to the current expectation that a single spermatozoön suffices for the fertilization of an egg. This curve is prepared from data of experiments calculated to bring eggs and sperm together in the freshest possible condition of the sperm. In general measured

quantities of washed eggs were put in measured amounts of sea-water, and measured quantities of definitely calibrated sperm suspensions added and stirred in as uniformly as possible.



CURVE I.

A control of unfertilized eggs in sea-water was always kept to guard against chance fertilizations. To illustrate: the last four determinations of the curve were made as follows: In four

crystallization dishes were placed 1,000 c.c. sea-water (*A*), 3,000 c.c. sea-water (*B*), 1,000 c.c. sea-water (*C*), 3,000 c.c. sea-water (*D*). To each was added 2 c.c. of a washed egg-suspension (about 3 per cent. to 5 per cent.). The sperm was then prepared as follows: (1) one drop dry sperm to 3.3 c.c. sea-water at 9.43 A.M. = 1 per cent.; (2) 1 c.c. of sperm 1 to 99 c.c. sea-water 9.43.30 A.M. = 1/100 per cent.; (3) 1 c.c. sperm 1 to 999 c.c. sea-water 9.45.30 = 1/1000 per cent. To *A* was added 1 drop sperm 2(1/100 per cent.) at 9.43.45; to *B* one drop sperm 2(1/100 per cent.) 9.44; to *C* one drop sperm 3(1/1000 per cent.) 9.45.45; to *D* one drop sperm 3(1/1000 per cent.) 9.45.45. An assistant stirred in the sperm thoroughly as added. The sperm concentration in *A* was therefore 1/100 × 1/30 × 1/1000 = 1/3,000,000 per cent.; in *B* it was 1/9,000,000 per cent.; in *C* 1/30,000,000 per cent.; in *D* 1/90,000,000 per cent. 1/3,000,000 per cent. falls between 21 and 22 on the scale, and the others as shown. The exact times of mixing the sperm are given because, as will appear beyond, time is an extremely important factor with reference to fertilizing power.

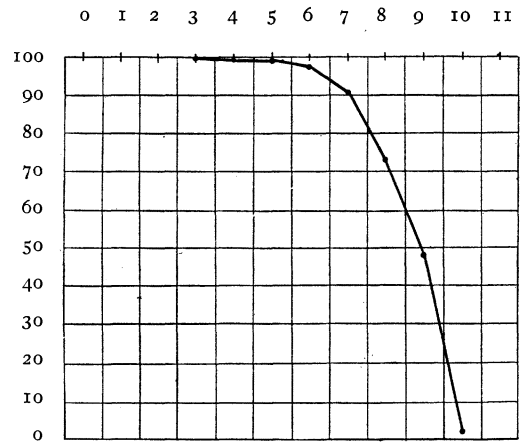
To appreciate the extent of this dilution it may be said that beyond a dilution of 1/10,000 per cent. (between 13 and 14 on the scale) one can rarely find a single spermatozoön in the jelly of the fertilized eggs. At about 1/2000 per cent. (11 on the scale) the sperm suspension does not even appear opalescent. We may therefore feel reasonably sure that beyond about 14 or 15 on the scale a single spermatozoön certainly suffices to completely fertilize an egg.

In further elucidation of the curve I may say that the critical (steep) part was covered by several determinations for each point. Thus there are five determinations averaged for the positions between 13 and 15. Seven between 15 and 18, five between 18 and 20, and six between 20 and 21. The determinations beyond 21 are single determinations. For the first part of the curve up to 13, there are numerous determinations. There are great variations in the single determinations compared with one another; these averages must therefore be regarded only as approximate values. With a sufficiently large number of determinations the irregularities between 15 and 17 and between 19

and 22 would no doubt disappear. But it is improbable that the general form of the curve would undergo any essential change even with a much more extensive series of determinations.

3. *Curves of Successive Half-dilutions.*

In contrast to these results, and for the purpose of defining the character of the main problem sharply, we may next consider the fertilizing power of a series of half dilutions of a 1 per cent. sperm suspension. The curves from these experiments furnish an almost incredible contrast to the one already given; as an example we may examine the following strikingly regular curve, Fig. 2. The first member of this series was a 1/8 per cent.



CURVE 2.

sperm suspension freshly prepared, thus falling in position 3 on the scale; 8 c.c. of this was taken (No. 1); to 4 c.c. of 1, 4 c.c. of sea-water was added (No. 2) = 1/16 per cent., $(1/2^4)$; to 4 c.c. of 2, 4 c.c. of sea-water was added (No. 3) = 1/32 per cent., $(1/2^5)$; this was continued eight places to $1/2^{10}$. Four drops of a 10 per cent. egg-suspension was then added to each, and the percentage of segmented eggs was counted three hours later. Plotted they give the above curve. In this case it will be seen that the fertilizing power almost ceases at $1/2^{10}$ = 1/1024 per cent. sperm suspension. The eggs and sperm were not at fault

because a parallel control series, in which the same quantities of the same lot of eggs were first placed in the same quantities of sea-water and sufficient of the original 1 per cent. sperm suspension added to make similar sperm dilutions, showed over 95 per cent. cleavage in each case, and actually 99 per cent. in No. 8 of the control where the sperm dilution was 1/1200 per cent. As a further control it may be added that eggs which fail to fertilize in such relatively concentrated sperm suspensions may all be fertilized by the subsequent addition of a trace of perfectly fresh sperm.

The type of experiment just cited was the first undertaken, and for a time it seemed to offer an almost insoluble problem, though the real explanation turned out to be extremely simple. I have twenty curves from similar experiments, fourteen of which run out absolutely from the third to the twelfth place on the scale (*i. e.*, from 1/8 per cent. to 1/4096 per cent.); in the remaining 6 (as in the curve just given) the dilutions were not carried far enough to reach the zero point, but they agree in principle with the others.

A number of control experiments demonstrated the relative lack of significance of the actual sperm concentrations. As one of these I may mention experiment C of August 3. In this case a series of sperm dilutions in powers of 4 was made from 1 per cent. The proportion of eggs fertilized ran off to 1 per cent. at $1/4^5$ ($1/2^{10}$) and to 0 at $1/4^6 = 1/4096$ per cent. But one drop of a 0.1 per cent. suspension of the original 1 per cent. sperm added to eggs in 200 c.c. sea-water fertilized 94 per cent. of them (control for sperm). Thus the control fertilized almost perfectly at 1/60,000 per cent. dilution, whereas the fifth member of the series of dilutions $1/2^{10}$ (1/1024 per cent. sperm) fertilized only 1 per cent. The actual concentration of the sperm is thus not the most significant thing.

This is also brought out strikingly in the following experiment (August 14). A series of half sperm dilutions was made as usual (Series *A*) 2 c.c. in each dish; to a second series (series *B*) of dishes was added 2 c.c. sea-water each and 4 drops of an egg-suspension. The numbers of series *B* were then inseminated by one drop each of sperm from the corresponding number of *A*, thus

diluting the sperm about 1/60. To each of the *A* series (except 1) four drops of the same egg-suspension was then added. The resulting percentages of fertilization are given in Table I.

TABLE I.

A.		*B*.	
1. (1%)	–	1. (1/60%)	– 99%
2. (1/2%)	– 96.5%	2. (1/120%)	– 99.5%
3. (1/4%)	– 99.5%	3. (1/240%)	– 98%
4. (1/8%)	– 99%	4. (1/480%)	– 60.5%
5. (1/16%)	– 98.5%	5. (1/960%)	– 51%
6. (1/32%)	– 96.5%	6. (1/1920%)	– 8.5%
7. (1/64%)	– 21%	7. (1/3840%)	– 1.5%
8. (1/128%)	– 6%	8. (1/7680%)	– 0
9. (1/256%)	– 3.5%	9. (1/15360%)	– 0

If we compare *A* and *B* in this table it will be seen that while it is true that *B* runs out earlier than *A*, nevertheless the fertilizations in the two series are not proportional to concentrations of sperm; for instance *A* 9 at 1/256 per cent. fertilizes 3.5 per cent. of the eggs, whereas *B* 3 at 1/240 per cent. fertilizes 98 per cent., *B* 4 at 1/480 per cent. fertilized 60.5 per cent., *B* 5 at 1/960 per cent. fertilizes 51 per cent. It is obvious that it is not concentration but *condition* of the sperm that is significant, which comes out with extreme emphasis in a control of this series. In this control, 1 drop of *A* (1 per cent. sperm) was added to 8 drops of the same egg suspension in 1,000 c.c. of sea-water 2 minutes after the other inseminations, thus making a 1/30,000 per cent. (1 × 1/1000 × 1/30) sperm suspension; every egg fertilized; the percentage of cleavage was 100 per cent.

The question then arises, what is this condition of the sperm which causes such loss of fertilizing power? We may note the following points: (1) To bring out the lack of significance of the absolute concentration of the sperm, in several of the experiments with successive half dilutions, counts were made of the numbers of spermatozoa seen in the egg-jelly of members of the series with no fertilizations: Thus on July 16 a series of half dilutions ran out to 0 in the seventh crystal (1/128 per cent. sperm): in ten eggs selected at random from this crystal, an average of 9 spermatozoa was counted in the jelly and in contact with the membrane of these eggs; but, as the upper and lower surfaces

could not be examined, the whole number must have been at least double; in No. 8 of the series, an average of five spermatozoa was counted with each egg; in No. 9 an average of 1.2; No. 10, 1.4; No. 11, 0.9. Similar counts were made in other cases. But in fertilizations under optimum conditions all of the eggs may fertilize in dilutions of sperm so great that it is almost impossible to find spermatozoa in the jelly of the eggs. (2) The spermatozoa are active and the eggs readily fertilizable in such a series as the above. Repeated observations were made on this point; which would be tedious to relate in detail.

It may be noted that in the fertilization under optimum conditions the eggs were first placed in sea-water, and given quantities of sperm then added; whereas in the experiments with successive dilutions eggs were added to sperm suspensions already made up. This suggested that the order of adding eggs and sperm might be of significance in some way. However, this does not appear to be the case.

The possibility remained that the repeated handling of the sperm in successive dilutions decreased their motility. Microscopical examination did not confirm this idea; and subsequent experiments disproved it, as the fundamental factor at least.

Thus it would appear that the only real difference between the optimum and minimum conditions of the fertilizing power of sperm dilutions is a time factor; under what I have called the optimum conditions the final dilution is made from a relatively concentrated sperm suspension in the presence of eggs; but under the conditions of successive dilutions time elaspses before the eggs are added.

Thus in Curve 2 the preparation of the series of sperm dilutions from the original 1 per cent. suspension occupied 22 minutes before the eggs were added. In Curve 1, on the other hand, less than a minute elasped from the time of preparing the 1/100 per cent. and 1/1000 per cent. sperm suspensions used in the last four determinations to the time of their use in inseminating (see p. 234); and the final dilution was made in the presence of the eggs.

The time factor is the real explanation as will be shown immediately. But at first sight it did not seem a very probable

explanation for two reasons: in the first place the time from preparation of the original 1 per cent. sperm suspension to that of addition of eggs is usually less than twenty-five minutes, which is usually considered too short a time for injury to sperm; and in the second place, after the addition of eggs to the sperm-dilution series, in several control experiments the original 1 per cent. sperm suspension was shown to be capable of fertilizing at 1/30,000 of 1 per cent. ($1/2^{15}$ ca.) by addition to eggs in sea-water. *If the sperm suspensions lose their fertilizing power with time, it must be that the significance of time in this respect varies inversely to concentration.* As soon as such a proposition is formulated it is easily tested experimentally, and this was done in a thorough fashion.

4. *Time as a Factor in the Fertilizing Power of Sperm Suspensions of Different Concentrations.*

The experiments under this head were performed in three ways:

A. A considerable quantity of the sperm suspension to be tested was made up, and divided in several equal parts in a series of bowls; measured equal quantites of the same egg-suspension were then added to members of the series at definite time intervals. This method was followed for sperm dilutions from 1/300 per cent. (between 8 and 9 in the scale) down. *B*. Measured amounts of the more concentrated sperm suspensions were added at time intervals to measured quantities of eggs in equal amounts of sea-water. *C*. Finally, to control the data in section 3, a series of sperm suspensions, made by successive half dilutions as in section 3, was divided in two equal series, and eggs were added at once to the one series, and after a time interval to the second.

A. The following table gives the data under method *A*. The figures at the head of each vertical column give the sperm dilution in fractions of the 1 per cent. sperm suspension; below is given the place of such a sperm suspension in the scale of powers of 2. The figures in the columns give the percentages of fertilizations for inseminations made at the time (age of the suspensions in minutes) indicated at the left. To illustrate the method of experimentation for one column which will serve for all the rest,

TABLE II.

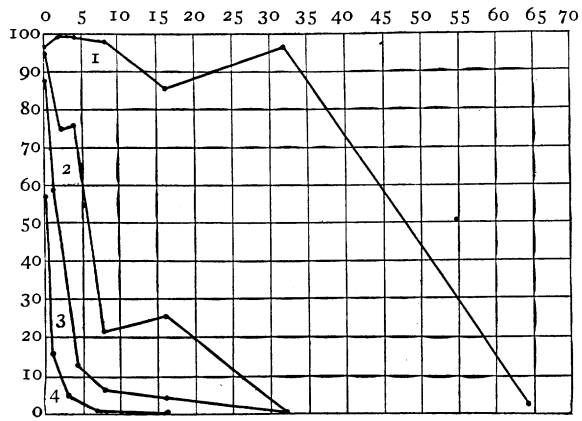
SHOWING RATE OF LOSS OF FERTILIZING POWER OF SPERM SUSPENSIONS.

	1/300	1/600	1/1200	1/2400	1/3000	1/3600	1/6000	1/12000	1/18000	1/24000	1/30000	1/60000	1/120000	1/240000
0	96	100	99.5	95	95.5	97	91	97	....	97.5	88	91.5	58	40
1	....	....	99.5	49.5	.....	52	.....	....	....	.....	....	42	16	6
2	99.5	98.5	.....	....	75	....	80	92.5	98	99.5	59.5	....	.....	....
3	....	....	100	91.5	.....	88.5	.....	.....	....	.....	....	....	4	16
4	99.5	95.5	.....	....	76	....	34.5	93.66	85.5	49.5	13	15.5	.....	....
6	....	....	93.5	52	.....	80.5	.....	.....	....	.....	....	....	0.5	1
8	98.5	65.5	.....	....	21	....	28.5	54.5	73.5	67	7	17	.....	....
16	85.5	?	99.5	45	26	16.5	.....	.....	14	36	4.5	1	0	0
32	96.5	67.5	30.5	1.5	0	4.5	9	1	0	4	0	0.5	0	0
64	2.5	19	0	0.5	0.5	3.5	2	0	0	4	0	0	0	0
120	8.5	3	.....	....	.....	....	.....	.....	....	.....	....	....	.....	....
	8–9	9–10	10+	11+	11–12	12−	12–13	13–14	14+	14–15	15+	16−	16–17	18−

we shall give the experimental data for 1/30,000 per cent.: August 18, 1914. The eggs of two females were taken at 9.50 A.M. and washed at 10.04, 10.06 and 10.23 (150 c.c. of sea-water being used in each washing). A series of 7 Syracuse crystals was then laid out with 10 c.c. of sea-water in each. To 1 was then added 5 drops of the egg-suspension. A single drop of fresh dry sperm was then added to 333.3 c.c. sea-water at 10.37 making a 1/100 per cent. sperm suspension, and 1 drop of this was added to crystal 1 at 10.37.30 and stirred in by an assistant making a 1/30,000 per cent. (1/100 × 1/10 × 1/30) sperm suspension in presence of eggs. One drop of the 1/100 per cent. sperm was also added to crystals 2–7, which contained no eggs, at 10.38, making 1/30,000 per cent. sperm suspension in each. To No. 2, 5 drops of the same egg-suspension was added at 10.40, to No. 3 at 10.42, to No. 4 at 10.46, to No. 5 at 10.54, to No. 6 at 11.10, to No. 7 at 11.42. At 2.30 P.M. my assistant, Mr. Cohn, then estimated the percentages of segmented eggs in each crystal, by first thoroughly mixing the eggs, then assembling them, taking a sample, and making two counts of 100 each, which were averaged.

The table shows (1) that the effect of time up to 64 minutes is to diminish the fertilizing power of the suspensions at every dilution represented. (2) That the rate of loss of fertilizing

power increases with dilution, *i. e.*, the effect of time varies inversely to concentration of sperm. This is brought out very clearly by the following curves (Fig. 3) of loss of fertilizing



CURVE 3.

power of sperm suspensions at different concentrations. The abscissæ represent age of sperm suspensions in minutes; the ordinates represent fertilizing power as expressed in percentages of segmenting eggs. Each curve stands for a given sperm dilution. Curve 1 represents loss of fertilizing power of a 1/300 per cent. sperm suspension, curve 2 of a 1/3000 per cent., curve 3 of a 1/30,000 per cent. and curve 4 of a 1/120,000 per cent. sperm suspension.

B. On August 6, I prepared a series of seven sperm dilutions in powers of 4 from 1 per cent. to 1/4096 per cent. Each of these was then used to fertilize a measured quantity of egg-suspension at the intervals given in Table III.

For the fertilizations 10 c.c. sea-water was measured out in advance in Syracuse crystals and 5 drops of a 5 per cent. egg suspension added to each. For each fertilization 1 drop of sperm was added and stirred in. It will be observed that 1 per cent. sperm lost none of its fertilizing power so far as this test went; 1/4 per cent. fell off from 96.5 per cent. to 16.3 per cent.; 1/16 per cent. from 46.5 per cent. to 0; 1/64 per cent. from 0.5 per cent. to 0 in the second place; whereas the greatest dilutions did

TABLE III.

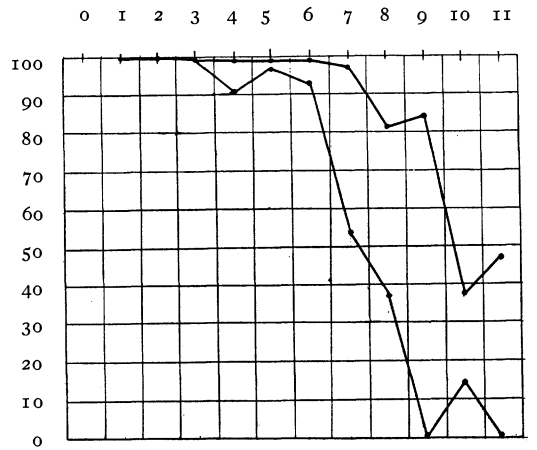
Sperm Dilution.	Made at	Fertilizations.				
		A.	*B.*	*C.*	*D.*	*E.*
		2.50 P.M.	3.01 P.M.	3.16 P.M.	3.35 P.M.	4.03 P.M.
1. 1%.....	2.20 P.M.	99 %	100%	100 %	99.5%	99.5%
2. 1/4%.....	2.28 P.M.	96.5%	68%	61.5%	18.5%	16.3%
3. 1/16%....	2.30 P.M.	46.5%	18%	8.5%	4 %	
4. 1/64%....	2.32 P.M.	0.5%	0%	0%	0 %	
5. 1/256%...	2.34 P.M.	0	0%	0%		
6. 1/1024%..	2.36 P.M.	0	0%			
7. 1/4096%..	2.39 P.M.					

not fertilize at all. It should of course be noted that the sperm suspensions used were diluted 300 times in the actual insemination (10 c.c. = 300 drops of sea-water, and one drop sperm added).

Thus time is an important factor in the fertilizing power of sperm dilutions from 1/4 per cent. down. The matter cannot be stated with great accuracy, but we can say in general (referring to Table II.) that sperm suspensions from 1/300 per cent. to 1/1200 per cent. lose their fertilizing power nearly completely in 64 minutes; from 1/2400 to 1/24,000 in 32 minutes; from 1/30,000 to 1/60,000 in 16 minutes; from 1/120,000 to 1/240,000 in 6 minutes. Table III. shows loss of fertilizing power of higher concentrations by a different method.

C. We are now in a position to understand the principal reason why the curves of successive half or quarter dilutions of a 1 per cent. sperm suspension run off so rapidly. The reason is that the preparation of the series requires time, 10 to 20 or more minutes. In the early experiments of this kind the significance of brief periods of time was not recognized, and so no time records were kept; but I have 13 curves with accurate time records. Of these I reproduce only two (Fig. 4). For this experiment (Sept. 5), (1) 6.6 c.c. of 1 per cent. sperm was prepared, 9.30 A.M. (2) 4 c.c. of 1 was transferred to a Syracuse watch crystal and 4 c.c. of sea-water added (= ½ per cent.). (3) 4 c.c. of 2 was transferred to crystal 3 and 4 c.c. sea-water added (= ¼ per cent.) and this was continued to 12 numbers (finished at 9.39.30 A.M.). The suspensions 2–12 was then divided in two equal amounts of 2 c.c. each, making series *A* and *B*. To each crystal of series *A* 2 drops of a 1 per cent. egg-suspension were

added at 9.47, that is, 17 minutes after the 1 per cent. sperm was prepared. Twenty-eight minutes later (10.15 A.M.) two drops of the same egg suspension were added to series *B*. The only difference between series *A* and *B* is the time factor. The percentages of cleavages were counted for both series, and the plotted results given the curves. Considerable loss of fertilizing power



CURVE 4.

has occurred in series *B* as compared with series *A*. Now, if we compare these time intervals with those given in Table II. we see that, in the curve of series *B* the last number, which is a $1/2^{11}$ or 1/2048 per cent. sperm suspension, loses its fertilizing power completely in 36 minutes (*i. e.*, from 9.39.30 to 10.15.30) which corresponds very well with the rate of loss of fertilizing power in a 1/2400 per cent. sperm suspension. This agreement is rather closer than usual; in some cases the series of ½ dilutions ran out at higher concentrations in about the same time; but in no case, I think, did they require more time. This suggests some possible stimulating effect of the successive changes which causes the spermatozoa to lose their fertilizing power more rapidly than under the time factor alone.

Gemmill (1900) observed that the duration of vitality of spermatozoa of sea-urchins and limpets tested by their movements or by the fertilizing capacity, varies greatly "according to the amount of sperm used in proportion to the volume of sea-

water in which it was shed." "When a small quantity of sperm was mixed with a large quantity of sea-water, the duration of vitality of the spermatozoa is short, but when the converse proportions are used, it is greatly lengthened." "By taking sperm from a sea-urchin and mixing it in different vessels with different quantities of sea-water, one obtains sets of spermatozoa, which will retain their vitality for a rising series of terms, *e. g.*, 8, 12, 16, 24, 48 and 72 hours. For the longest term, the proportion of spermatic fluid to sea-water should be not less than 1 to 10."

Gemmill was thus dealing with the same phenomenon with which we are concerned. He gives, however, no exact quantitative data and relatively few experiments were performed. He attributes the results to (1) greater activity of the spermatozoa, and consequent earlier exhaustion in the more dilute suspensions and (2) to dilution of the "spermatic fluid" by which he supposes the spermatozoa to be nourished.

5. *Other Factors in the Fertilizing Power of Sperm Suspensions.*

In the large number of experiments carried out to test the fertilizing power of sperm suspensions the general form of the curves is remarkably constant. Some, however, are quite irregular, and it was never possible to get *exactly* the same curve in the repetition of any experiment. A few of the irregularities may conceivably be due to error, as for instance the accidental presence of some toxic substance in one of the dishes of a series, though painstaking care was used to avoid such sources of error. The failure to obtain exactly the same curve in different experiments is no doubt also due in part to the natural variability of different lots of eggs and sperm.

In an attempt to discover the sources of variation and error, the effect of egg concentration, *i. e.*, the absolute quantity of eggs in a given bulk of a sperm suspension of given strength was tested. On the whole the effect of egg-concentration was found to be relatively small within so wide a range that it cannot be regarded as a large factor in the variability of the curves; because the egg-concentration of the curves was always below the point where it was demonstrably a limiting factor. Tests were made of sperm suspensions ranging from 1/62.5 per cent. to 1/8,000

per cent. But it was only from about 1/500 per cent. down that any considerable effect was observed within the range of egg concentration employed.

The method of the experiments tabulated (Table IV.) may be given for 1/500 per cent. sperm as it was the same for the others August 31: A quantity of 1/500 per cent. sperm suspension was freshly prepared 10.54.30 A.M., 2 c.c. of this was then placed in each of seven crystals (1–7). From 10.56.30 to 10.59 A.M. eggs were added as follows: to 1, one drop of a 1.75 per cent. egg-suspension, to 2 two drops, to 3 four drops, to 4 eight drops, to 5 sixteen drops, to 6 one c.c., to 7 two c.c. The numbers in the table give the percentages of segmented eggs. The tests with 1/1,000 per cent. and 1/2,000 per cent. sperm were made with the same egg suspension. For the tests with 1/4,000 per cent. and 1/8,000 per cent. sperm a 3.3 per cent. egg-suspension was used. Thus for each series the egg-concentration is approximately doubled in successive numbers of the series (in No. 7 = 64 times No. 1).

TABLE IV.

EFFECT OF EGG-CONCENTRATION ON THE FERTILIZING POWER OF SPERM SUSPENSIONS.

Sperm Suspensions.	Egg-suspensions.						
	1.	2.	3.	4.	5.	6.	7.
1/500%	100	99	99.5	97	93.5	82.5	56
1/1000%	97.5	94.5	93	76.5	48?	76.5	33.5
1/2000%	96.5	83.5	75	72.5	42.5	36	32
1/4000%	79.5	66.5	42.5	47.5	12.5	16	4.5
1/8000%	46.5	52	80?	30.66	27	15.5	7.5

The percentages of fertilization fall off in each of these sperm suspensions with increase of egg-concentration, and the amount of falling off increases in general with the dilution of the sperm. There was certainly no numerical deficiency of spermatozoa in the highest egg-concentrations; the reason for the falling off therefore appears rather obscure, and as it is not involved in the present problem, I shall not discuss it here. But as the egg-concentration employed in any of the preceding experiments did not exceed that of column 3, and the same egg-concentration

was always employed throughout any experiment, it is obvious that the effect to be attributed to the egg-concentration employed in the preceding experiments is very small.

III. Discussion.

Within a wide limit of egg-concentration the important factors in fertilizing power of sperm suspensions are: (1) concentration, (2) time. A third factor, which is not of equal significance to the other two, is the given variability of the reproductive elements. Such variability attaches of course both to ova and spermatozoa; in general it will affect only absolute values for given combinations, and not at all the relative values found in any single experiment. Moreover, as it is a chance factor, it will tend to be eliminated in a series of determinations. Fortunately both eggs and spermatozoa of *Arbacia* are relatively very constant materials if care be taken to wash the eggs thoroughly, and if the factors of concentration and time are fairly constant for the sperm. For the eggs these two latter factors are of such slight importance within the given limits as to be practically negligible. The significance of the concentration factor for the fertilizing power of sperm is of course obvious without discussion. We therefore turn to the time factor.

The most significant aspects of the time factor are, first, the unexpectedly rapid rate of loss of fertilizing power of sperm suspensions, and second the increase of rate of loss with dilution. There are but two ways of explaining these facts: either (1) the motility of the spermatozoa is quickly reduced in sperm suspensions to such an extent that they cannot bore into the egg or (2) the spermatozoa lose some substance essential for the fertilization reaction.

The following are the objections to the first alternative: (*a*) Microscopical examination lends it no support; I have repeatedly observed, that fertilizing power of sperm cannot be expressed either in terms of motility, or of success in penetrating the jelly of the egg and coming in contact with the membrane. In the experiments on successive half dilutions (p. 238) I kept records, in several series, of the numbers of spermatozoa in the jelly of unfertilized eggs, and found in some cases an average of 9

spermatozoa visible in the jelly, or on the membrane of certain lots of eggs none of which had fertilized; this could not be more than half of the spermatozoa in association with such eggs; and other observations made immediately after insemination demonstrated the high degree of motility of spermatozoa of entirely barren sperm suspensions.[1]

These observations contrast in the most striking manner with the fact that not a single spermatozoön can be seen in the jelly of eggs fertilized with highly dilute fresh sperm suspensions, where, nevertheless, nearly every egg may be fertilized.

(*b*) Penetration of the egg is not solely a function of motility of the spermatozoön. Penetration follows, as a matter of fact, after the fertilization reaction has begun, and it is due to the inception of such reaction, not the reverse as is commonly assumed.[2] In *Nereis*, as I have previously described, penetration does not take place until 45 to 50 minutes after insemination and the initiation of the fertilization reaction. The facts described in this paper show that in *Arbacia* no penetration takes place unless the sperm has started the fertilization reaction; if this does not take place, the spermatozoön remains external, however active it may be. And if it does occur the initiating spermatozoön is speedily engulfed by the egg.

(*c*) It is not easily understood on this theory why dilute sperm suspensions should lose their fertilizing power more rapidly than

[1] Glaser's experiments (1915) would bear the interpretation that, in those cases of normal insemination described by him in which fertilization does not occur except in the presence of several spermatozoa for each egg, the time factor which I have just described was operative. In other words that the majority of the spermatozoa in question had lost their receptors. But in the absence of exact data as to age and concentration of the sperm suspension, it cannot be asserted that this is the correct interpretation although I obtain exactly the same results in my time series (p. 238). My dilution experiments prove beyond a doubt that a single spermatozoon suffices for the whole process of fertilization under optimum conditions (defined on p. 233). Glaser's experiments, however, raise the question whether the efficacy of heavy insemination in the case of a stale sperm suspension is due to mass action, or to the survival of a small percentage of effective spermatozoa? So far as I can see this question can not be answered on the basis of our present information.

[2] Spermatozoa may penetrate into unripe ovocytes in some cases, as has been noted by several observers; in such a case there is no fertilization reaction. In the present experiments the unfertilized eggs were not penetrated by the spermatozoa.

more concentrated suspensions;[1] the relative freedom from CO_2 and other sperm excreta should favor a longer continuation of their motility in the dilute suspensions rather than the reverse.

(*d*) Moreover, in general the results of recent fertilization studies such as the antagonistic action of sperm suspensions of different phyla, inhibition of fertilization in the presence of blood of the species, or in the absence of certain ions (Loeb, '14), or again the sterility in certain self-fertilizations, and finally the inability of spermatozoa to penetrate fertilized eggs, unite in demonstrating the relative lack of significance of motility as such.

We come therefore to the conclusion that the individual spermatozoa in suspension tend to lose their fertilizing material, so that an increasing proportion of these spermatozoa become absolutely ineffective whatever their motility. This conclusion is in agreement with all the data of the foregoing experiments, and seems to be the only one competent to explain the results.

The following questions arise: (1) Whether the loss of this substance by the sperm is a mere process of diffusion or an active secretion? (2) Can the substance be recovered from the fluid of the suspension, or can its presence in the fluid be demonstrated in any way?

As regards the first question: In the case of the ova we know that the external jelly-covering is loaded with sperm-agglutinating substance which diffuses into the sea-water continuously. It is theoretically possible, at least, to apply a similar conception to the spermatozoön, although no such covering is demonstrable. The more rapid loss of fertilizing power in the greater dilutions would be consistent with this interpretation. From this point of view we would have to regard the sperm head as covered superficially with a layer of fertilizing material, like the phosphorus on a match. Such a conception is by no means impossible. On the other hand the fact that dilutions reached by a series of successive half-dilutions from 1 per cent. lose their

[1] Gemmill (1900) observed the same phenomenon and concluded that the more rapid exhaustion of spermatozoa in dilute suspensions is due to dilution of a hypothetical nutritive medium which keeps the spermatozoa of concentrated suspensions in a vigorous condition. This explanation comes back to the principle of loss of motility, so far as it relates to fertilizing power.

fertilizing power more rapidly than the same dilutions made in one stroke, indicates that successive stimulation hastens the loss, which therefore appears more in the nature of a secretion or a discharge than mere diffusion. The source of the substance must ultimately be the sperm cell itself, and it is quite possible that, as in the case of the egg, there is both a superficial layer and an internal supply.

It must be admitted that the data are inadequate to answer this problem. The statement of the problem can therefore serve only to bring out the resemblance between the spermatozoön and the ovum in respect to the existence of a fertilizing substance in each, the fertilizin in the case of the ovum and the sperm receptors in the case of the spermatozoön, and also the possible resemblance in respect to the disposition of the substances in each. It certainly is an interesting parallelism that both cells contain a substance necessary to fertilization, which may be lost in the sea-water.

The most interesting and crucial question of course concerns the possibility of detecting this lost substance in the fluids of the suspensions. If such a substance actually occurs in the fluid it should have the property of fertilizing ova; unless it can be detected by this property, we have no other indicator for it. So far I have not been able to make even a beginning on this problem. As is well known a number of experimenters have attempted without success to derive a fertilizing medium from spermatozoa. It has been suggested by Loeb that the reason for the failure to secure an extract of spermatozoa that will fertilize is that the motile power of the spermatozoön is needed to carry the effective substance into the egg. But it may equally well be that the methods hitherto employed have been too brutal; the substance may well be too labile to withstand extraction by ether, etc.

My results strongly suggest, if they do not prove, that such a substance must be present in the fluid of sperm suspensions of *Arbacia*, and they therefore suggest other methods for securing it for testing. We must bear in mind that it can form only an extremely small proportion of the entire spermatozoön, as proved by morphological considerations alone, and that it must

be superficial in position and easily detached as proved by its effectiveness before the spermatozoön penetrates. Extracts of the entire spermatozoön must contain numerous other substances which may neutralize its effectiveness.

The difficulty of the investigation as shown by my experiments is that it is liberated only very slowly in concentrated suspensions and that its amount in dilute suspensions would presumably be too slight to be effective. Some means can probably be devised for liberating it in concentrated sperm suspensions and freeing it of the spermatozoa for testing.

Finally I may point out that the conclusion that spermatozoa lose a substance necessary for the exercise of their fertilizing power is consistent with my own point of view of the mechanism of fertilization as well as with Loeb's. From my point of view the spermatozoön loses its receptors, viz., the substance that activates the fertilizin of the egg; from Loeb's point of view the spermatozoön loses its lysin, the substance that corrodes (cytolyzes) the egg.

My previous experiments had shown that eggs produce a certain substance in sea-water (fertilizin) which is necessary for their fertilization; fertilized eggs no longer produce this substance and are incapable of fertilization. Both eggs and spermatozoa therefore contain substances, more or less liable to loss, which are necessary for fertilization. The mechanism of fertilization cannot possibly, therefore, be regarded in the simple manner postulated by Loeb's theory. The existence of parthenogenesis demonstrates the efficacy under given conditions of the egg-substance alone; we must therefore regard the spermatic substance essentially as an activator of the fertilizin of the egg.

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